Cell, Vol. 88, 833–843, March 21, 1997, Copyright ©1997 by Cell Press

Notch Activity Influences the αβ versus γδ T Cell Lineage Decision

Tracy Washburn,* Edina Schweighoffer,‡ Thomas Gridley,† David Chang,* B. J. Fowlkes,‡ Dragana Cado,* and Ellen Robey*

*Department of Molecular and Cell Biology
University of California
Berkeley, California 94720

†The Jackson Laboratory
Bar Harbor, Maine 04609

‡Laboratory of Cellular and Molecular Immunology
National Institute of Allergy and Infectious Disease
National Institutes of Health
Bethesda, Maryland 20892

Summary

The choice between the αβ or γδ T cell fates is influenced by the production of functional, in-frame rearrangements of the *TCR* genes, but the mechanism that controls the lineage choice is not known. Here, we show that T cells that are heterozygous for a mutation of the *Notch1* gene are more likely to develop as γδ T cells than as αβ T cells, implying that reduced Notch activity favors the γδ T cell fate over the αβ T cell fate. A constitutively activated form of Notch produces a reciprocal phenotype and induces thymocytes that have functional γδ*TCR* gene rearrangements to adopt the αβ T cell fate. Our data indicate that Notch acts together with the newly formed T cell antigen receptor to direct the αβ versus γδ T cell lineage decision.

Introduction

During development, the choice between alternative cell fates is often controlled by direct cell-to-cell contact between equivalent precursor cells. In many cases, this involves signaling through the transmembrane receptor Notch and insures that initially equivalent precursor cells adopt distinct developmental fates. The Notch receptor plays a crucial role in development in organisms as diverse as Drosophila and mouse.

The mechanism by which Notch controls the choice of cell fate has been extensively studied in the formation of the peripheral nervous system in Drosophila (Heitzler and Simpson, 1991). In this case, Notch and its ligand, Delta, are both expressed on precursor cells that are choosing between primary and secondary cell fates. Mutation of either *Notch* or *Delta* leads to the appearance of extra primary fate cells (neurons) at the expense of secondary fate cells (epidermal cells), whereas expression of a constitutively active form of Notch leads to extra secondary fate cells at the expense of primary fate cells. While heterozygous mutations of either *Notch* or *Delta* have no affect on peripheral nervous system development, when precursor cells that are heterozygous for *Notch* develop next to wild-type cells, the heterozygous cells adopt the primary fate and their wild-type neighbors adopt the secondary fate. These results suggest the existence of a feedback mechanism that amplifies small differences in Notch signaling between neighboring precursor cells that are choosing between primary and secondary fates.

The mechanism driving this feedback loop has been further explored in C. elegans (Wilkinson et al., 1994). In this example, the two equivalent precursor cells that are choosing between the primary anchor cell (AC) fate and the secondary ventral uterine precursor (VU) cell fate both express the Notch-like receptor, LIN-12, and its ligand. Over time, one cell gradually begins to express more of the ligand and less of the receptor and its neighbor does the opposite, until eventually one cell expresses only the receptor and its neighbor expresses only the ligand. These observations suggest that signaling through LIN-12 leads to down-regulation of the ligand and up-regulation of the receptor, resulting in a self-reinforcing feedback loop. In this way, initially small differences in the levels of Notch and its ligand on precursor cells become amplified over time, such that eventually one cell becomes the "receiving" cell and its neighbor becomes the "sending" cell. Recent evidence suggests that a similar feedback mechanism might also exist in vertebrates (Chitnis et al., 1995; Girard et al., 1996; Robey et al., 1996).

Although this feedback mechanism can amplify a small difference in Notch signaling between neighboring precursor cells, the issue of how this initial difference is generated is still unclear. In the case of the AC versus VU decision, the choice of which cell adopts the primary or secondary fate appears random, and it has been suggested that a random fluctuation in the relative levels of receptor and ligand might begin the process (Wilkinson et al., 1994). In other examples, however, the choice between primary and secondary cell fates is controlled by other environmental signals (Beitel et al., 1995; Katz et al., 1995), raising the possibility that these signals might regulate the levels of Notch and/or its ligand on precursor cells. The question of how the Notch signaling pathway is integrated with other developmental cues, however, is as yet unresolved.

We are exploring how Notch works together with other signals to specify cell fate in the mammalian immune system. During thymic development, developing T cells, or thymocytes, rearrange and express the genes encoding their antigen receptors (T cell antigen receptor, or TCR). The type of antigen receptor expressed by a developing T cell ultimately determines the type of mature T cell it will become (reviewed in Robey and Fowlkes, 1994). Several mammalian Notch homologs have been described (Coffman et al., 1990; Weinmaster et al., 1991; del Amo et al., 1992; Stifani et al., 1992; Weinmaster et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994), and at least one of these homologs, Notch1, is expressed in developing T cells (Weinmaster et al., 1991; Weinmaster et al., 1992; Hasserjian et al., 1996; Robey et al., 1996). We have recently shown that Notch activity affects the choice of T cell precursors between the CD4 and CD8 T cell fates (Robey et al., 1996). Because much is known about the control of T cell fate by the T cell antigen receptors, the thymus represents an excellent context in which to investigate the interplay between Notch and other signals that guide cell fate decisions.

In this report, we examine the role of Notch and the T cell antigen receptor in the αβ versus γδ T cell lineage decision. The majority of T cells express a T cell antigen receptor consisting of α and β chains. These αβ T cells include $CD4^+$ helper T cells and $CD8^+$ killer T cells that regulate the immune response and help to fight intracellular pathogens. A smaller, distinct lineage of T cells expresses a T cell antigen receptor consisting of γ and δ chains. In addition to expressing a distinct form of the antigen receptor, these γδ T cells generally do not express CD4 or CD8 and have distinct functions compared to αβ T cells (reviewed in Bluestone et al., 1991; Allison, 1993).

At an early stage of thymic development, T cell precursors first begin to rearrange their β, γ, and δ *TCR* genes. Recent evidence suggests that the decision of the precursor to become a γδ or an αβ lineage T cell is influenced by whether or not it successfully rearranges its *TCR*β or its γ and δ *TCR* genes. In immature T cells, the product of the rearranged *TCR*β gene pairs with a surrogate version of TCRα, called preTα, and this preTCR complex is thought to permit the developmental progression of αβ lineage T cells (Mombaerts et al., 1992a; Saint-Ruf et al., 1994; Fehling et al., 1995). This developmental progression includes expression of CD4 and CD8, proliferation, and up-regulation of *TCR*α locus rearrangement. The δ and γ *TCR* genes are also rearranged in αβ T cells, and these rearrangements are selectively depleted of in-frame rearrangements (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995). These observations suggest that successful completion of γδ*TCR* gene rearrangement diverts precursor cells from the αβ lineage into the γδ lineage (Allison and Lanier, 1987; Pardoll et al., 1987).

While it appears that *TCR* gene rearrangements influence the αβ versus γδ lineage decision, there are also indications that there is more to the story. For example, mutation of either *TCR*β or *preT*α substantially reduces, but does not abolish, the development of $CD4^+CD8^+$ thymocytes, suggesting that a signal through the preTCR complex is not an absolute requirement for αβ T cell development. Moreover, if expression of an in-frame γδ*TCR* invariably led to γδ T cell development, one might expect a complete block in αβ T cell development in mice expressing a rearranged γδ*TCR* transgene. This, however, is not the case: many γδ*TCR* transgenic mice contain significant numbers of αβ lineage T cells (Bonneville et al., 1990; Dent et al., 1990; Sim et al., 1995; Schweighoffer and Fowlkes, 1996). It has also been shown that high expression of a rearranged γδ*TCR* transgene in the absence of *TCR*β gene rearrangements leads to the appearance of a small population of $CD4^+CD8^+$ thymocytes, suggesting that in some situations a γδ*TCR* can actually promote αβ T cell development (Kersh et al., 1995). Finally, there are reports that the presence of normal thymocytes (Shores et al., 1990) or γδ T cells (Lynch and Shevach, 1993) can induce the expression of CD4 and CD8 in a small proportion of thymocytes that cannot rearrange their own *TCR* genes, suggesting that the thymic environment can influence the development of $CD4^+CD8^+$ thymocytes. Thus, it appears that other factors, in addition to the presence of in-frame β or γδ *TCR* rearrangements, influence whether T cell precursors adopt the αβ or γδ T cell fate.

Here, we provide evidence that Notch participates in the αβ versus γδ T cell lineage choice and that Notch activity favors the αβ T cell lineage. Our data support a model in which the presence of in-frame β or γδ *TCR* gene rearrangements influences whether or not a thymic precursor receives the Notch signal, and the Notch signal, in turn, promotes development along the αβ T cell pathway.

Results

A Reduction in *Notch1* Gene Dosage Favors the γδ T Cell Fate over the αβ T Cell Fate

Previous studies have shown that Notch1 is expressed in developing thymocytes (Weinmaster et al., 1991; Weinmaster et al., 1992; Hasserjian et al., 1996; Robey et al., 1996). Moreover, the highest level of Notch1 is expressed in $CD4^-CD8^-$ thymocytes (Hasserjian et al., 1996; Robey et al., 1996), a subset that contains precursor activity for both αβ and γδ lineage T cells (Petrie et al., 1992; Godfrey et al., 1993). This expression pattern, together with indications that Notch plays a role in many different cell fate decisions, suggests that Notch could participate in the αβ versus γδ T cell lineage decision.

To investigate this possibility, we examined the effect of a reduction in *Notch1* gene dosage on T cell development. Mice that lack both copies of the *Notch1* gene arrest in development at around day 9 of gestation (Swiatek et al., 1994; Conlon et al., 1995), precluding an examination of T cell development in these mice. Therefore, we exploited the findings of Heitzler and Simpson (1991), who showed that the relative expression levels of Notch on adjacent developing cells influence cell fate decisions. In their studies of the developing Drosophila peripheral nervous system, wild-type cells adopted the epidermal fate (the secondary fate) when neighboring cells expressed lower Notch levels, but adopted the neural fate (the primary fate) when neighboring cells expressed higher Notch levels. Presumably, a feedback loop, such as that observed in C. elegans development (Wilkinson et al., 1994), amplifies initial differences in the expression of Notch on neighboring cells. We therefore investigated whether interactions between developing T cells that are heterozygous for *Notch1* ($N1^{+/-}$) or wild-type ($N1^{+/+}$) might influence the choice of T cell fate.

To create an environment where $N1^{+/-}$ cells develop adjacently to $N1^{+/+}$ cells, we reconstituted the immune system of irradiated *rag1* mutant mice with a mixture of equal portions of fetal liver or bone marrow cells from $N1^{+/+}$ and $N1^{+/-}$ donor mice. Because the *rag1* mutant mice lack mature T and B cells (Mombaerts et al., 1992b; Spanopoulou et al., 1994), the immune systems of these chimeric mice are derived exclusively from the donor stem cells. The two donors have allelic differences at the *Ly5* locus (Ly5.1/5.2 and Ly5.2/5.2), allowing us to distinguish the two donor types by using a monoclonal antibody that recognizes Ly5.1. After 1–5 months to allow reconstitution of the immune system, we analyzed the chimeric mice to determine what fraction of lymphocytes was derived from the $N1^{+/-}$ or $N1^{+/+}$ donors.

Data from 38 such mixed genotype chimeric mice are shown in Figure 1. $N1^{+/-}$ and $N1^{+/+}$ stem cells contribute equally to the B cell compartment (Figure 1A, mixed genotype chimeras). This suggests that heterozygosity

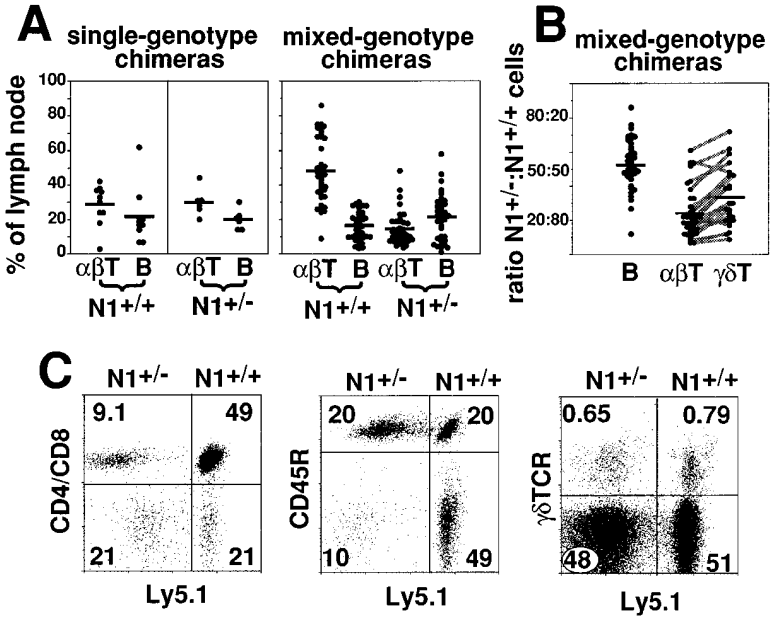

Figure 1. A Reduction in *Notch1* Gene Dosage Inhibits αβ T Cell Development

50:50 mixtures of donor hemopoietic stem cells from *Notch1*$^{+/-}$ and *Notch1*$^{+/+}$ littermates were used to reconstitute the immune system of irradiated *rag1* mutant mice. The two donor types differed at the *Ly5* locus. (A) Compiled data from 38 chimeras, in which the two donors differed at the *Notch1* locus (mixed genotype chimeras) and from 8 chimeras in which the two donors differed at the *Ly5* locus, but had the same *Notch1* genotype (single genotype chimeras). The number of αβ T cells or B cells derived from each donor type is plotted as a percentage of total lymph node cells from a chimeric mouse. The bars represent the average, and the dots are values from individual chimeras. For the mixed genotype chimeras, the averages are: *N1*$^{+/+}$ αβ T cells, 48% (3.03); *N1*$^{+/+}$ B cells, 16% (1.35); *N1*$^{+/-}$ αβ T cells, 14% (1.55); and *N1*$^{+/-}$ B cells, 21% (2.13). For the single genotype chimeras, the averages are: *N1*$^{+/+}$ αβ T cells, 28% (3.7); *N1*$^{+/+}$ B cells, 22% (5.06); *N1*$^{+/-}$ αβ T cells, 30% (3.25); and *N1*$^{+/-}$ B cells, 20% (2.44). Standard errors of the mean are given in parentheses.

(B) The relative effect of the *Notch1* mutation on B cells, αβ T cells, and γδ T cells in mixed genotype chimeras. The ratio of *Notch1*$^{+/-}$ donor to *Notch1*$^{+/+}$ donor in each population is indicated. The data for T and B cells are replotted from Figure 1A. The contribution of *Notch1*$^{+/-}$ stem cells to the B cell, αβ T cell, or γδ T cell subsets was analyzed from the lymph nodes or spleens of mixed genotype chimeras. The average contribution of *N1*$^{+/-}$ cells to each population is: B cells, 53%; αβ T cells, 24%; and γδ T cells, 32%. Connecting lines indicate values that come from the same chimeric mouse. In order to statistically compare values from individual chimeras, we divided the percent contribution of the *N1*$^{+/-}$ donor to the γδ T cell population by the percent contribution of the *N1*$^{+/-}$ donor to the αβ T cell population for each chimera. If *N1*$^{+/-}$ donor cells contribute equally to γδ and αβ populations, the log of this ratio is expected to be 0. The actual value is 0.136 with a standard error of 0.027, indicating that *N1*$^{+/-}$ donor cells contribute significantly more to the γδ T cell population than to the αβ population. (C) Representative flow cytometric analysis of lymphocytes from a mixed genotype chimera using a marker for donor type (Ly5.1) in conjunction with a marker for αβ T cells (CD4 and CD8), a marker for B cells (CD45R), or a marker for γδ T cells (γδTCR). Lymph node cells, or B cell–depleted spleen cells, were used for the analysis of γδTCR expression. In this particular chimera, the *N1*$^{+/+}$ donor was Ly5.1/Ly5.2 and the *N1*$^{+/-}$ donor was Ly5.2/Ly5.2. However, in approximately half of the chimeras, the Ly5.1 marker was expressed by the *N1*$^{+/-}$ donor.

at the *Notch1* locus does not affect the ability of stem cells to repopulate irradiated hosts or to produce mature B cells. In contrast, the *N1*$^{+/-}$ stem cells produced three times fewer αβ T cells than did *N1*$^{+/+}$ donor stem cells (Figure 1A, mixed genotype chimeras; representative FACS [fluorescence-activated cell sorting] plots from an individual mixed genotype chimeric mouse are shown in Figure 1C). These data suggest that thymocytes expressing relatively higher levels of Notch1 are more likely to enter the αβ T cell lineage than are cells expressing lower levels of Notch1.

To establish that *N1*$^{+/-}$ stem cells are not intrinsically deficient in producing αβ T cells, we examined the number of αβ T cells produced by *N1*$^{+/-}$ stem cells when *N1*$^{+/+}$ stem cells are absent. Intact *N1*$^{+/-}$ mice have the same ratio of αβ T cells to B cells as their wild-type littermates (data not shown). In addition, chimeric mice repopulated with 50:50 mixtures of donor stem cells of the same *N1* genotype (differing at the *Ly5* locus) produced normal numbers of αβ T cells, regardless of the *N1* genotype of the donors (Figure 1A, single genotype chimeras). Thus, the difference in the production of αβ T cells is observed only when *N1*$^{+/+}$ and *N1*$^{+/-}$ cells develop together in the same mouse. This suggests that the reduction in *N1*$^{+/-}$ αβ T cells in the mixed genotype chimeras results not from intrinsic defects in the development of *N1*$^{+/-}$ αβ T cells, but from a cell fate choice imposed on them through a Notch-mediated interaction with neighboring *N1*$^{+/+}$ cells.

αβ and γδ T cells arise from a common progenitor. The observation that reduced *N1* gene dosage inhibits αβ T cell development raises the possibility that progenitors with only one copy of the *Notch1* gene are more likely to choose the γδ T cell lineage over the αβ T cell lineage than their wild-type neighbors. To investigate this possibility, we examined chimeric mice repopulated with 50:50 mixtures of *N1*$^{+/-}$ and *N1*$^{+/+}$ stem cells to determine the proportion of *N1*$^{+/-}$ cells within the γδ and αβ T cell lineages (Figure 1B). As shown earlier, the *N1*$^{+/-}$ cells make up on average only 24% of the αβ T cell population, although they represent an equal proportion of the starting stem cells population and make up 53% of the B cell compartment (Figure 1B; data replotted from Figure 1A). *N1*$^{+/-}$ cells also represent a minority of the γδ T cell population (32%). However, when the *N1*$^{+/-}$ contributions to the αβ and γδ T cell populations from individual chimeras are compared (see lines connecting data from the same chimeric mouse, Figure 1B), it is evident that the *N1*$^{+/-}$ donor cells contributed more on average to the γδ T cell population than to the αβ T cell population (21 out of 25 chimeras). These data are consistent with the hypothesis that stem cells that have lower levels of Notch than their neighbors are more likely to choose the γδ lineage over the αβ lineage.

We next investigated whether early thymocyte development exhibited the same Notch-dependent cell fate bias that we observed in mature T cell populations. We

examined two developmental stages, an immature thymocyte population that contains precursor activity for both the γδ and αβ T cell lineages (interleukin-2 receptor [IL-2R]$^+$CD4$^-$CD8$^-$; Petrie et al., 1992; Godfrey et al., 1993) and a thymocyte population that represents αβ lineage thymocytes of an intermediate stage of maturity (CD4$^+$CD8$^+$) (Figure 2). In the thymus of mixed genotype chimeric mice reconstituted with 50:50 mixtures of *N1*$^{+/-}$ and *N1*$^{+/+}$ stem cells, the *N1*$^{+/-}$ donor cells contributed, on average, 28% to the IL-2R$^+$CD4$^-$CD8$^-$ population and 15% to the CD4$^+$CD8$^+$ population (Figure 2A; representative FACS plots from an individual mixed genotype chimeric mouse are shown in Figure 2B). The *N1*$^{+/-}$ cells in each of the 8 chimeras contributed less to the CD4$^+$CD8$^+$ population than to the IL-2R$^+$CD4$^-$CD8$^-$ population (see lines connecting individual chimeras). These data support the notion that *N1*$^{+/-}$ precursor cells are less likely to enter the αβ T cell lineage than are their *N1*$^{+/+}$ neighbors.

Within the T cell lineage, *N1*$^{+/-}$ cells contribute more to the γδ population than to the αβ population; however, *N1*$^{+/-}$ stem cells produce fewer γδ T cells overall than do wild-type cells that develop in the same mice. One possible explanation for this discrepancy is that *Notch1* is involved in an earlier cell fate decision in the hemopoietic lineage and the *N1*$^{+/-}$ stem cells are diverted into an earlier, as yet unidentified, fate. This is consistent with the observation that *N1*$^{+/-}$ cells contribute only 28% to the T cell precursor population that gives rise to αβ and γδ T cells. An alternative possibility is that heterozygosity of the *Notch1* locus gives cells a competitive disadvantage for proliferation or survival compared to wild-type cells that develop in the same mouse. In order to distinguish between these possibilities, we examined the effect of an activated form of Notch on the αβ versus γδ lineage decision.

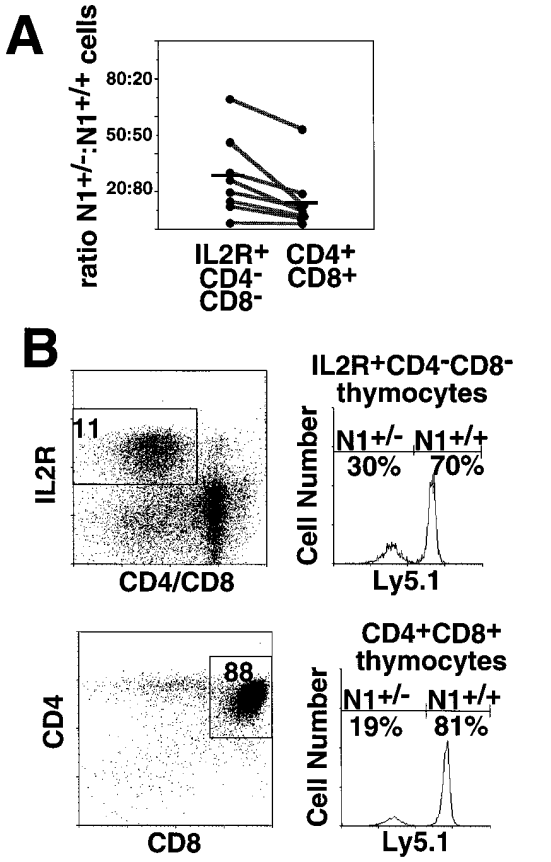

Figure 2. A Reduction in *Notch1* Gene Dosage Inhibits the Production of CD4$^+$CD8$^+$ Thymocytes

Thymocytes from chimeric mice reconstituted with 50:50 mixtures of *N1*$^{+/+}$ and *N1*$^{+/-}$ hemopoietic stem cells were analyzed for expression of CD4, CD8, IL-2R, and Ly5.1.

(A) Compiled data from 8 radiation chimeras showing the relative contribution of the *Notch1* mutant stem cells to IL-2R$^+$CD4$^-$CD8$^-$ subsets and CD4$^+$CD8$^+$ subsets. The bars represent the averages, and the dots are values from individual chimeric mice. The average contribution to the IL-2R$^+$CD4$^-$CD8$^-$ population is 28% and to the CD4$^+$CD8$^+$ population is 15%. Connecting lines indicate values that come from the same chimera. In order to compare values from individual chimeras statistically, we divided the percent contribution of the *N1*$^{+/-}$ donor to the CD4$^+$CD8$^+$ thymocyte population by the percent contribution of the *N1*$^{+/-}$ donor to the IL-2R$^+$CD4$^-$CD8$^-$ population for each chimera. If *N1*$^{+/-}$ donor cells contribute equally to these two populations, the log of this ratio is expected to be 0. The actual mean of 0.287 with a standard error of 0.05 is significantly different from the value of 0 that would be expected if the *Notch* mutation were affecting these two populations equally.

(B) Representative flow cytometric analysis of thymocytes from hemopoietic stem cell chimeras. For analysis of IL-2R expression, thymocytes were depleted of CD8$^+$ thymocytes and stained with anti-IL-2R, anti-CD4, anti-CD8α, and anti-Ly5.1 antibodies. For analysis of CD4 and CD8 expression, total thymocytes were stained with anti-CD4, anti-CD8, and anti-Ly5.1 antibodies. Rectangles indicate the gates used to define IL-2R$^+$CD4$^-$CD8$^-$ and CD4$^+$CD8$^+$ subsets.

An Activated Form of Notch Favors the αβ T Cell Fate

If reduced Notch activity favors the γδ T cell fate over the αβ T cell fate, then increased Notch activity should have the reciprocal effect. We have previously described transgenic mice that express an activated form of Notch in developing thymocytes (Robey et al., 1996) and have shown that activated Notch affects a cell fate decision within the αβ T cell lineage: the choice of αβTCR$^+$ CD4$^+$CD8$^+$ precursors between the CD4 and CD8 T cell fates. Because the activated *Notch* transgene is under the control of the Lck proximal promoter (Chaffin et al., 1990), it should be expressed early in thymic development, perhaps at the time of the αβ versus γδ lineage decision. We therefore investigated the possibility that the activated *Notch* transgene might also influence the choice between the αβ and γδ T cell fates.

The choice of uncommitted precursors between the αβ and γδ T cell lineages is controlled in part by *TCR* gene rearrangements. Successful completion of γ and δ *TCR* gene rearrangements favors the γδ T cell fate (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995), whereas successful completion of β *TCR* gene rearrangement promotes αβ T cell development (Mombaerts et al., 1992a, 1992b; Shinkai et al., 1992). Although mice expressing the activated *Notch* transgene *(NotchIC* transgenic mice) have approximately normal numbers of γδTCR$^+$ thymocytes, there is a 3.7-fold increase in the proportion of γδTCR$^+$ thymocytes that express CD8 (Figures 3A and 4C). We also examined

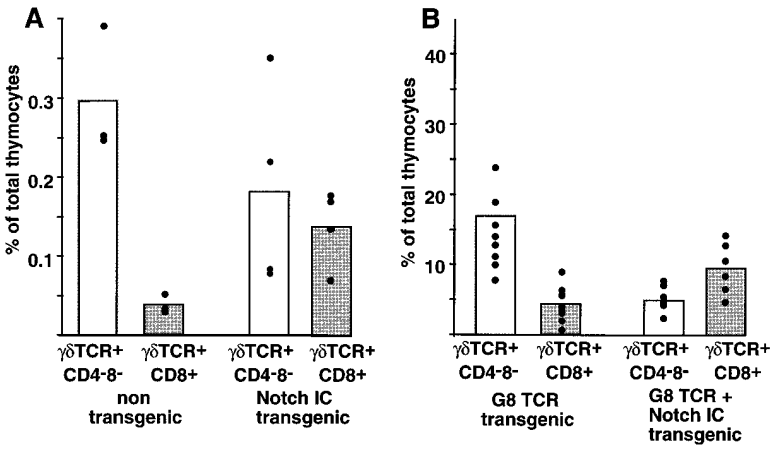

Figure 3. An Activated Form of *Notch* Leads to the Appearance of $CD8^+$ Thymocytes Bearing γδTCRs

(A) Thymocytes from mice bearing an activated *Notch* transgene were analyzed for expression of γδTCR, CD4, and CD8.

(B) *NotchIC* transgenic mice were crossed with mice carrying a rearranged γδ*TCR* transgene, *G8 TCR.*

Thymocytes from mice expressing either the *G8 TCR* transgene or both the *G8 TCR* and *NotchIC* transgenes were analyzed for expression of γδTCR, CD4, and CD8. The number of $\gamma\delta TCR^+$ thymocytes that express neither CD4 nor CD8 (open bars) or that express CD8 and/or CD4 (closed bars) is plotted as the percentage of total thymocytes. The bars represent the average values, and the dots are values from individual mice. $CD8^+$ thymocytes in *G8 TCR* transgenic mice virtually all express both CD8β and CD8α and consist of $CD4^+CD8^+$ as well as immature $CD4^-CD8^+$ thymocytes (data not shown). Mature $CD4^-$ $CD8^+$ thymocytes are not present due to the absence of β2-microglobulin in these mice (see Experimental Procedures).

the effect of activated Notch on the development of thymocytes expressing a rearranged γδ*TCR* transgene. Mice bearing the *G8* γδ*TCR* have increased numbers of $\gamma\delta TCR^+$ thymocytes, 80% of which express neither CD4 nor CD8 (Dent et al., 1990; Schweighoffer and Fowlkes, 1996; Figure 3B). In contrast, in mice bearing both the *G8* γδ*TCR* and the *NotchIC* transgene, the majority of the $\gamma\delta TCR^+$ thymocytes express CD8. Although some activated γδ T cells and intestinal intraepithelial γδ T cells express CD8, they express the CD8αα homodimeric form of CD8 and do not express CD4 (reviewed in Bluestone et al., 1991). In contrast, the $\gamma\delta$ TCR^+ thymocytes that we observe in *NotchIC* transgenic mice express CD8αβ heterodimer, and a significant fraction coexpress CD4 (data not shown). Because CD4 and CD8αβ heterodimer expression are generally associated with αβ lineage T cells, this raises the possibility that, in the presence of activated Notch, thymocytes that successfully rearrange their γ and δ *TCR* genes might adopt the αβ T cell fate rather than the γδ T cell fate.

If thymocytes that express a γδTCR and activated Notch can develop as αβ T cells, then activated Notch might drive αβ T cell development in *TCR*β mutant mice. Successful rearrangement and expression of *TCR*β is necessary for normal αβ T cell development, and mutation of the *TCR*β gene results in an approximately 100-fold reduction in the number of $CD4^+CD8^+$ cells in the thymus (Mombaerts et al., 1992a; Figure 4A). Strikingly, in *TCR*β mutant mice that express activated Notch, the number of $CD4^+CD8^+$ thymocytes is restored to almost normal levels (Figures 4A and 4B). This increase reflects both an increase in the total number of thymocytes and an increase in the percentage of $CD4^+CD8^+$ cells in these mice (Figure 4B). Activated Notch could promote the development of $CD4^+CD8^+$ thymocytes by inducing precursors to choose the αβ lineage or by enhancing the survival or proliferation of existing $CD4^+CD8^+$ thymocytes. However, we have previously shown that expression of the activated *Notch* transgene in an otherwise wild-type mouse does not alter the proliferation or life span of $CD4^+CD8^+$ thymocytes (Robey et al., 1996). Our results, therefore, are most compatible with the interpretations that activated Notch influences the choice of precursor cells between the αβ versus γδ lineages and that activated Notch can promote the development of $CD4^+CD8^+$ in the absence of the TCRβ chain.

Mice that are mutant for *TCR*β can still rearrange and express their γ and δ *TCR* genes. This raises the possibility that the development of $CD4^+CD8^+$ thymocytes in *NotchIC* transgenic, *TCR*β mutant mice might depend upon expression of a functional γδ TCR. To address this question, we examined the effect of activated Notch in mice that lack expression of all *TCR* genes due to a targeted disruption of the *rag1* gene (Spanopoulou et al., 1994). *rag1* mutant mice display an early and complete block in T cell development and have no detectable $CD4^+CD8^+$ cells in the thymus (Mombaerts et al., 1992b; Spanopoulou et al., 1994; Figure 4A). Introduction of the activated *Notch* transgene into *rag1* mutant mice has no apparent effect on thymic development, and $CD4^+CD8^+$ thymocytes remain undetectable (Figure 4A). Thus, while an activated form of Notch can overcome the block in αβ T cell development in *TCR*β mutant mice, it cannot drive αβ T cell development in the absence of *TCR* gene rearrangement.

$CD4^+CD8^+$ Thymocytes from *NotchIC* Transgenic, *TCR*β Mutant Mice Carry In-Frame Rearrangements of the *TCR*δ Locus

The ability of activated Notch to restore $CD4^+CD8^+$ development in *TCR*β mutant mice, but not in *rag1* mutant mice, suggests that expression of a γδ TCR may be necessary to drive αβ T cell development in *TCR*β mutant mice. While the majority of thymocytes in *NotchIC* transgenic, *TCR*β mutant mice does not express γδ TCR on their surfaces, it is still possible that these cells expressed a γδ TCR at the time that they were choosing between the αβ and γδ T cell lineages. There are indications that the γ and δ *TCR* loci are transcriptionally inactive in αβ lineage T cells (discussed in Raulet et al., 1991; Livak et al., 1995). Thus, it is possible that an immature T cell might make a functional γδ TCR, respond to the constitutive Notch signal by developing as an αβ T cell, and then turn off expression of the γδ TCR. To investigate this possibility, we asked whether thymocytes of

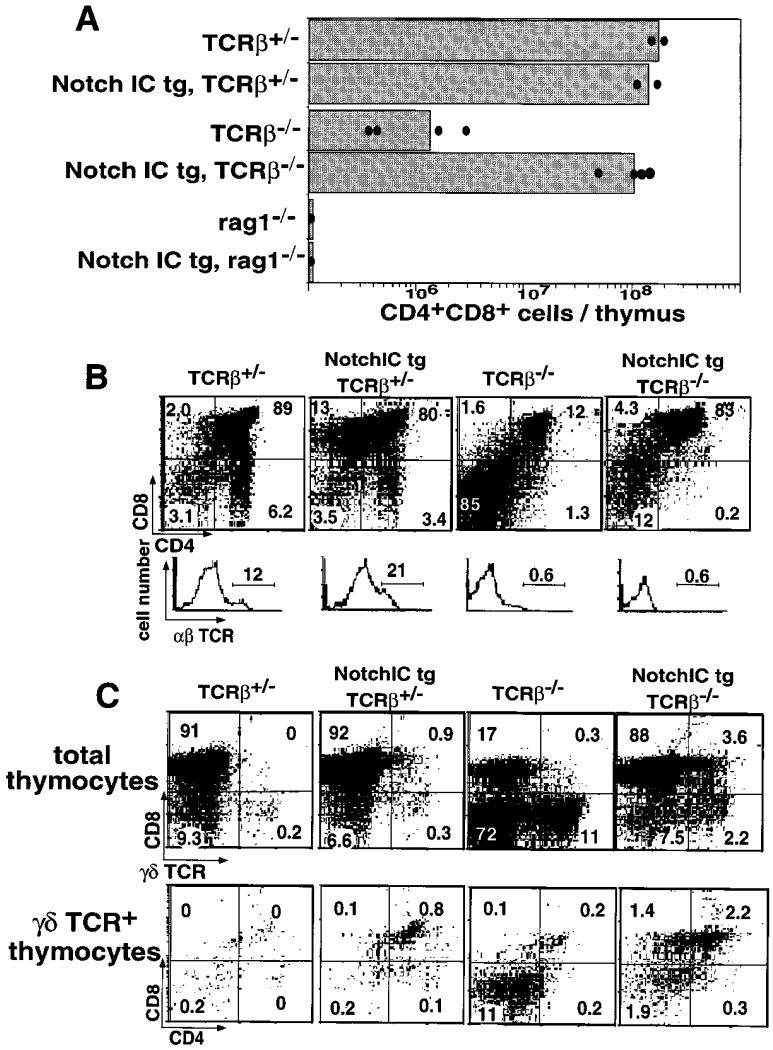

Figure 4. An Activated Form of Notch Promotes the Development of $CD4^+CD8^+$ Thymocytes in *TCR*β Mutant Mice, but Not in *rag1* Mutant Mice

Mice bearing an activated *Notch* transgene were crossed with mice carrying a germline disruption of the *TCR*β gene or the *rag1* gene. Thymocytes from the second mutant backcross were analyzed by flow cytometric analysis as described in Experimental Procedures.

(A) Total number of $CD4^+CD8^+$ thymocytes in mice of the indicated genotypes. Each dot represents the value from an individual mouse, and bars represent average values.

(B and C) Representative FACS plots of CD4, CD8, αβTCR, and γδTCR on thymocytes from mice of the indicated genotypes. Numbers indicate the percentage of total thymocytes within the indicated gates.

NotchIC transgenic, *TCR*β mutant mice carry functionally rearranged *TCR*δ loci.

A method termed PCR–RFLP analysis (Dudley et al., 1995) was used to determine whether thymocytes in *NotchIC* transgenic, *TCR*β mutant mice carry in-frame *TCR*δ rearrangements. In this procedure, DNA encoding rearranged V–J junctional regions from T cell populations are amplified by PCR, digested with restriction enzymes, end-labeled with ^{32}P, and resolved by size on denaturing acrylamide gels. Populations in which the *TCR* locus is functionally rearranged show a prominent band every third base, corresponding to in-frame rearrangements of the V and J segments, whereas populations in which there is a selection against functional rearrangements show reduced bands in the in-frame third positions. The *TCR*δ locus is nested inside the *TCR*α locus (Chien et al., 1987); therefore, thymocytes that attempt to rearrange their α *TCR* loci delete the δ *TCR* loci from the chromosome. However, the deleted DNA persists in thymocytes and mature resting T cells and can be readily detected using PCR (Dudley et al., 1995; Livak et al., 1995). We analyzed Vδ4–Jδ1 junctions from thymocytes of *NotchIC* transgenic, *TCR*β mutant mice (Figures 5A and 5B). Thymocytes from *TCR*$\beta^{+/-}$ mice and *TCR*β mutant mice were included for comparison. As previously observed, thymocytes from *TCR*β mutant mice show a selectively in-frame pattern of Vδ4–Jδ1 rearrangements (Dudley et al., 1995; Figures 5A and 5B), consistent with the fact that there are proportionally more $\gamma\delta TCR^+$ thymocytes in these mice (Mombaerts et al., 1992a; Figure 4C). In contrast, *TCR*$\beta^{+/-}$ mice show a more even distribution of rearrangements including out-of-frame Vδ4–Jδ1 rearrangements (Dudley et al., 1995; Figure 5). Thymocytes from *NotchIC* transgenic, *TCR*β mutant mice display predominantly in-frame rearrangements, comparable to the pattern seen in *TCR*β mutant mice. This suggests that functional *TCR*δ rearrangements were selected in thymocytes of *NotchIC* transgenic, *TCR*β mutant mice.

Because the majority of thymocytes in *NotchIC* transgenic, *TCR*β mutant mice are $CD4^+CD8^+$ (Figure 4B), we suspected that most of the Vδ4–Jδ1 rearrangements detected in these samples came from $CD4^+CD8^+$ thymocytes. To confirm this, we isolated $CD4^+CD8^+\gamma\delta TCR^-$ thymocytes from these mice by fluorescence-activated cell sorting and performed additional PCR–RFLP analysis on the sorted population, this time using primers corresponding to Vδ5–Jδ1. Once

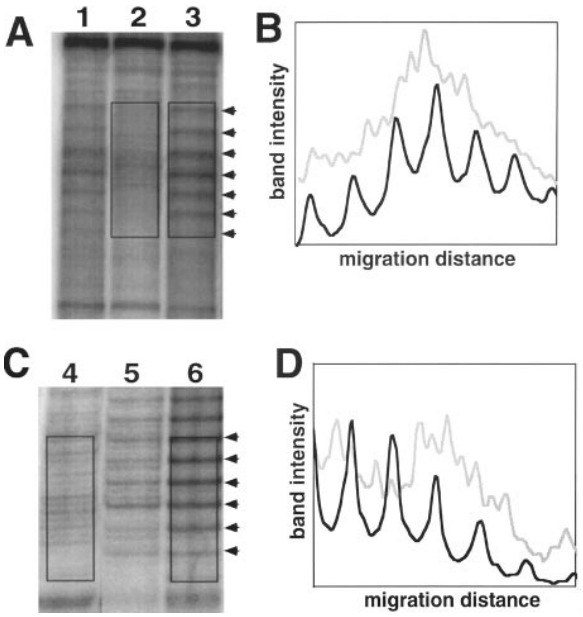

Figure 5. The *TCR* δ Locus Is Selectively In-Frame in Thymocytes from *NotchIC* Transgenic, *TCR*β Mutant Mice

(A) Genomic DNA from thymocytes was amplified using a Vδ4–Jδ1 primer pair, digested with HinfI, end-labeled with ^{32}P, and resolved on a denaturing acrylamide gel. DNA was isolated from total thymocytes of *TCR*β mutant mice (1), *TCR*β$^{+/-}$ mice (2), and *NotchIC* transgenic, *TCR*β mutant mice (3).

(B) PhosphorImager traces of lanes corresponding to *TCR*β$^{+/-}$ mice (gray line, lane 2) and *NotchIC-9* transgenic, *TCR*β mutant (solid line, lane 3).

(C) Genomic DNA from thymocytes was amplified using a Vδ5–Jδ1 primer pair, digested with HaeIII, end-labeled with ^{32}P, and resolved on a denaturing acrylamide gel. DNA was isolated from thymocytes of *TCR*β$^{+/-}$ mice (4), *TCR*β mutant mice (5), and sorted CD4$^+$ CD8$^+$γδTCR$^-$ thymocytes from *NotchIC* transgenic, *TCR*β mutant mice (6).

(D) PhosphorImager traces of lanes corresponding to *TCR*β$^{+/-}$ mice (gray line, lane 4) and sorted *NotchIC* transgenic, *TCR*β mutant (solid line, lane 6). The rectangles indicate the regions of the gel displayed in the PhosphorImager traces. Arrowheads indicate the position of in-frame rearrangements based on the pattern observed with *TCR*β mutant thymocytes and an adjacent sequencing ladder (not shown).

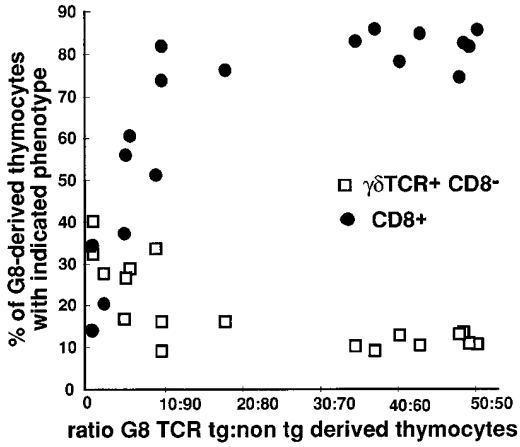

Figure 6. The Development of Thymocytes Bearing a Rearranged γδ *TCR* Transgene Can Be Influenced by Interactions with Neighboring Nontransgenic Thymocytes

Mixed hemopoietic stem cell chimeras were constructed using various proportions of donor cells from *G8 TCR* transgenic and nontransgenic Ly5.1 mice. After 5 weeks, thymocytes were analyzed for expression of Ly5.1, γδTCR, and CD8. The percentage of *TCR* transgenic–derived thymocytes that are γδTCR$^+$CD8$^-$ (open squares) or CD8$^+$ (closed circles) is plotted as a function of the ratio of *G8 TCR* transgenic–derived to nontransgenic-derived donors. All of the donor and host mice used in this experiment were β*2-microglobulin* mutant, in order to avoid negative selection of thymocytes expressing the *G8 TCR*. The input ratio of *G8 TCR* transgenic to nontransgenic stem cells was 10-fold higher on average than the resulting ratio, suggesting that the *G8 TCR* transgenic donor cells have a competitive disadvantage over nontransgenic donor cells.

again, a selectively in-frame pattern of *V*δ gene rearrangements was observed, confirming that, although these cells do not express γδ TCR, they have in-frame δ *TCR* rearrangements. We also examined rearrangement of Vγ1.1–Jγ4 on sorted CD4$^+$CD8$^+$γδTCR$^-$ thymocytes and found that they were also selectively in-frame (data not shown). Taken together, these data imply that the ability of activated Notch to drive CD4$^+$CD8$^+$ development in *TCR*β mutant mice is dependent on the presence of functional γδ receptor.

The Development of γδ Lineage Thymocytes Bearing a Rearranged γδ *TCR* Transgene Can Be Enhanced by Interactions with Nontransgenic Thymocytes

The observation that Notch activity can drive thymocytes bearing in-frame γδ*TCR* genes to develop along the αβ pathway, together with indications that the relative level of Notch1 signaling between precursors may influence the αβ versus γδ lineage choice, suggests that Notch signaling between thymocytes might normally limit the proportion of thymocytes that can develop as γδ T cells. This could explain the observation that mice expressing rearranged γδ *TCR* transgenes still have substantial numbers of αβ T cells. Perhaps Notch signaling between precursors induces some cells to adopt the αβ lineage, in spite of the fact that they express a functional γδ TCR. If this hypothesis is correct, we might expect that a thymocyte bearing a functional γδ TCR would be more likely to become a γδ T cell if the majority of its neighbors develop as αβ T cells.

To investigate whether nontransgenic thymocytes could influence the development of thymocytes bearing functional γδ *TCR* genes, we generated mixed hemopoietic stem cell chimeras using various ratios of stem cells from mice bearing the *G8* γδ*TCR* transgene and nontransgenic mice. In this experiment, nontransgenic donor cells bear an allelic marker (Ly5.1$^+$) allowing them to be distinguished from *G8 TCR* transgenic–derived cells (Ly5.1$^-$). In addition, we used β*2-microglobulin* mutant mice for both donor and host mice in these experiments to avoid negative selection on class I MHC (major histocompatibility complex) of thymocytes expressing the *G8 TCR* transgene (Dent et al., 1990). After 5 weeks to allow reconstitution of the thymus, we examined thymocytes by flow cytometry to determine the proportion of thymocytes derived from the *G8 TCR* transgenic donors and the fraction of these *G8 TCR* transgenic–derived thymocytes that develop as γδ lineage cells (Figure 6). We find that when thymocytes derived from transgenic donors make up less than 10% of total thymocytes, the proportion of *G8 TCR* transgene–derived

thymocytes that are γδTCR$^+$CD8$^-$ increases, whereas the proportion of *G8 TCR* transgenic–derived thymocytes that express CD8 is reduced. These CD8$^+$, *G8 TCR* transgenic–derived thymocytes (Ly5.1$^-$) resemble αβ lineage thymocytes in that they express CD8β as well as CD8α and are predominately CD4$^+$ and γδTCR$^-$ (data not shown). These data indicate that interactions between G8 TCR–bearing thymocytes limit the number of thymocytes that develop as γδ T cells and are consistent with the notion that Notch signaling between thymocytes can inhibit the γδ T cell fate.

Discussion

Although successful rearrangement of either β or γδ *TCR* genes may influence the lineage decision (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995), the mechanism by which thymocytes choose between the αβ and γδ lineage remains unknown. Here, we identify a new participant in the αβ versus γδ lineage decision: the transmembrane receptor Notch. Previous studies provide evidence for a feedback mechanism that amplifies small differences in the levels of Notch and its ligand on initially equivalent precursor cells (Heitzler and Simpson, 1991; Wilkinson et al., 1994). As a result, a cell that expresses less Notch than its neighbor ultimately avoids the Notch signal and adopts the primary fate, while its neighbor receives the Notch signal and adopts the secondary fate. Here, we provide evidence that the relative level of Notch1 on developing T cells can influence their fate, such that cells with a single copy of the *Notch1* gene are less likely to become αβ T cells than wild-type cells that develop in the same mouse. We also show that the ratio of γδ to αβ T cells is consistently higher in T cells derived from *Notch1*$^{+/-}$ stem cells compared to wild-type cells, suggesting that reduced Notch activity favors the γδ lineage over the αβ lineage. Finally, we show that expression of an activated form of Notch permits thymocytes with in-frame rearrangements of the γ and δ *TCR* genes to develop along the αβ pathway. These data indicate that Notch acts together with the newly formed TCR to direct the αβ versus γδ T cell lineage decision.

How do Notch and TCR act together to direct thymocytes to the αβ or γδ lineage? While we do not yet have a complete answer, the effect of the activated *Notch* transgene in *TCR*β mutant and *rag1* mutant backgrounds provides an important clue. Mutation of the *TCR*β gene leads to an early block in αβ T cell development, implying that rearrangement and expression of the *TCR*β gene is important for normal αβ T cell development. Introduction of an activated form of Notch into *TCR*β mutant mice restores CD4$^+$CD8$^+$ thymocytes to normal levels. This suggests that Notch signaling acts downstream or in parallel with TCRβ to direct cells to the αβ lineage (Figure 7). In wild-type mice, αβ lineage T cells have predominantly nonproductively rearranged γ and δ loci (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995), suggesting that successful γδ *TCR* gene rearrangement normally diverts precursors out of the αβ lineage and into the γδ lineage. Together, these observations suggest that the production of in-frame β or γδ *TCR* genes may influence whether or not a precursor receives a Notch signal.

How might productive *TCR* gene rearrangements influence Notch signaling? One intriguing possibility is suggested by the observation that T cells that develop in the same thymus can influence the fates of one another. For example, we show that the relative levels of Notch1 in thymocytes influences whether they develop as αβ or γδ T cells. We also show that thymocytes expressing a rearranged γδ*TCR* transgene are more likely to develop as γδ T cells when the majority of their neighbors develop as αβ T cells. Together, these observations suggest the existence of a Notch-mediated feedback mechanism that directs neighboring thymocytes to adopt distinct fates. According to this notion, thymocytes that successfully rearrange their γδ *TCR* genes might induce their neighbors to develop as αβ T cells via a Notch signal. Further investigations into how Notch activity is normally regulated in the thymus will be needed to test this model.

We show here that a constitutively activated form of Notch can direct thymocytes with productively rearranged γδ *TCR* genes to develop as αβ T cells. However, there are indications that even when Notch is regulated normally, not every cell that makes in-frame γδ *TCR* gene rearrangements avoids the Notch signal and adopts the γδ T cell fate. For example, we have shown that one can alter the proportion of T cells that adopt the αβ or γδ lineage simply by altering the relative *Notch1* gene dosage, a result that would not be expected if the lineage decision were controlled exclusively by *TCR* gene rearrangements. Moreover, previous studies show that expression of a rearranged γδ*TCR* transgene often does not abolish αβ T cell development (Bonneville et al., 1990; Dent et al., 1990; Sim et al., 1995; Schweighoffer and Fowlkes, 1996). This raises the possibility that signals through Notch and the newly formed TCRs act in concert to direct precursor cells to the αβ or γδ T cell lineage and that neither signal by itself can completely override the other.

At what point does a T cell precursor become committed to the αβ or γδ lineage? Analysis of a cell fate decision in C. elegans indicates that the choice of cell fate can be a gradual process that must be reinforced over time before cells are actually lineage committed (Wilkinson et al., 1994). In this light, it is interesting to consider that a thymocyte precursor might receive signals through the TCR and Notch that influence its lineage choice well before the cell actually becomes committed to either lineage. Indeed, it is possible that commitment to the γδ lineage does not occur until the thymocyte turns off the machinery needed to rearrange its antigen receptor genes, and thus can no longer rearrange the *TCR*β gene. Conversely, commitment to the αβ T cell lineage might not occur until a cell rearranges both of its *TCR*α loci, leading to the deletion of the *TCR*δ loci. Such a scenario would provide a prolonged period of time in which a precursor could assess the level of Notch signal and any signals through the TCR while choosing its fate.

Notch activity alone is not sufficient to direct a precursor cell to the αβ lineage. Rather, the precursor cell needs a signal from the TCR, in conjunction with the

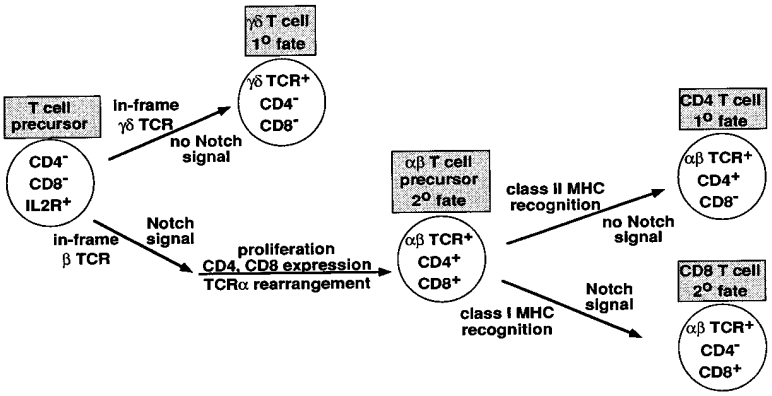

Figure 7. A Model for the Regulation of T Cell Fate by Notch and TCR Signals

A T cell precursor first attempts to rearrange its γ, δ, and β *TCR* genes. If the cell makes in-frame rearrangements of γ and δ *TCR,* this prevents the cell from receiving the Notch signal and it develops as a γδ T cell. If the cell makes an in-frame rearrangement of β *TCR,* it receives the Notch signal. This leads to proliferation, CD4 and CD8 expression, and rearrangement of the *TCR*α locus. This αβ T cell precursor then tests its αβTCR for recognition of MHC proteins on thymic epithelial cells. If its TCR recognizes class II MHC, the cell does not receive the Notch signal and it develops as a CD4 T cell. If its TCR recognizes class I MHC, the cell receives the Notch signal and develops as a CD8 T cell. A constitutively activated form of Notch causes developing T cells to misinterpret their developmental cues. In the presence of activated Notch, a cell that makes in-frame γδ TCR rearrangements still receives the Notch signal and develops along the αβ pathway, and a thymocyte whose αβTCR recognizes class II MHC still receives the Notch signal and develops along the CD8 pathway.

Notch signal, to develop along the αβ pathway. This result parallels our previous observations for the role of Notch in the CD4 versus CD8 T cell lineage decision (Figure 7; Robey et al., 1996). In that case, activated Notch can direct CD8 T cell development in the absence of class I MHC proteins, ligands recognized by the αβTCR that are normally required for CD8 T cell development. However, activated Notch is not sufficient to promote CD8 T cell development when both class I and class II MHC proteins are absent. Thus, for both the αβ versus γδ lineage decision and for the CD4 versus CD8 lineage decision, the TCR appears to be playing two distinct roles. On the one hand, a specific TCR signal normally directs the lineage decision and can be overridden by an activated form of Notch. On the other hand, some TCR signaling is required for maturation and/or survival, and this requirement cannot be overridden by activated Notch. The nature of these TCR signals and how they regulate Notch and promote T cell maturation are important questions for the future.

Experimental Procedures

Mice

Mice were bred and maintained in U.C. Berkeley and NIAID Research Animal Facilities according to AAALAC specifications. *Notch1* mutant mice (Swiatek et al., 1994) and *NotchIC-9* transgenic mice (Robey et al., 1996) have been previously described. *rag1* mutant mice (Spanopoulou et al., 1994) were kindly provided by David Baltimore. *G8* transgenic mice (Dent et al., 1990) that had been backcrossed five times to B10.D2 were kindly provided by Drs. J Bluestone and L. Matis. These mice were then crossed two times to β*2-microglobulin* mutant mice (previously backcrossed five times to C57Bl/10 mice). All experiments using the *G8 TCR* transgene were done using β*2-microglobulin* mutant mice, in order to avoid negative selection due to class I MHC (Dent et al., 1990). *TCR*β mutant mice (Mombaerts et al., 1992a) were purchased from Jackson Laboratories.

Hemopoietic Stem Cell Chimeras

Mice heterozygous for a disruption of the *Notch1* gene were crossed with C57Bl/6 mice congenic at the *Ly5* locus (Ly5.1). Offspring from this cross that were heterozygous for *Notch1 ($N1^{+/-}$, Ly5.1/Ly5.2)* were crossed with C57Bl/6 mice *($N1^{+/+}$, Ly5.2/5.2).* The offspring from this cross were used as donors of hemopoietic stem cells. Donors pairs were chosen that differed at the *Ly5* locus (*Ly5.2/Ly5.2* mixed with *Ly5.1/Ly5.2*) and that differed at the *Notch1* locus ($N1^{+/+}$ mixed with $N1^{+/-}$), except for "same genotype chimeras" in which donor pairs had the same *Notch1* genotype. Mice were typed for the presence of the *Notch1* insertion mutation by PCR, using a 5′ primer (5′-TGGGAAGACAATAGCAGGCATGC-3′) that anneals to sequences within the insertion and a 3′ primer (5′-CAGGGGTTGGA GAGACATTCATTG-3′, Swiatek et al., 1994) that anneals to the coding region of EGF repeat 33. The *Notch1* genotypes of 76% of the mice in this study were also examined by genomic Southern blotting as previously described (Swiatek et al., 1994), and in every case, the typing by Southern blot confirmed the PCR typing. Most of the donor pairs were siblings, and each in a pair was distinguished by an allelic difference at the *Ly5* locus (cross described above) or *Ly9* locus, which encodes another cell surface marker expressed on lymphocytes. We chose $N1^{+/-}$ donors expressing one *Ly5* allele (or *Ly9* allele) as often as expressing the other, thus eliminating any bias from unequal sampling of cell surface marker alleles. In addition, the majority of mixed chimeras reported in this study were generated using independent pairs of donor stem cells. Bone marrow chimeras were made according to standard protocols (Coligan et al., 1994). For *Notch1* mutant mixed chimeras, *rag1* mutant host mice (Spanopoulou et al., 1994) were irradiated with 1000 Rads from a Cs source and were intravenously injected 3–24 hr later with 10^7 donor cells. Fetal liver, 1 day postnatal liver, or adult bone marrow served as the source of donor hemopoietic stem cells. Bone marrow cells were T cell–depleted with anti-Thy1.2 (J1J) and anti-Lyt1.2 (C3PO) antibodies in combination with low toxicity rabbit complement (Cedarlane Labs, Westbury, NY). Host mice were analyzed 1–5 months postinjection. For *G8 TCR* transgenic chimeras, NK cells of the recipients were depleted by injecting PK136 antibody intraperitoneally the night before bone marrow transfer. Recipients were injected with 2×10^7 donor bone marrow cells intravenously, then 5 weeks after bone marrow transfer, the chimeric mice were sacrificed and the thymuses were removed and analyzed.

Flow Cytometry

Thymocyte and lymph node cell suspensions were prepared according to standard protocols (Coligan et al., 1994). For the analysis of IL-$2R^+$ thymocytes, thymocytes were first depleted of $CD8^+$ thymocytes using anti-CD8α antibodies and low toxicity rabbit complement (both from Cedarlane Labs, Westbury, NY). Live cells were recovered on a Ficoll-Paque gradient. Splenocytes were enriched for γδ T cells by passage over a nylon wool column. Cells were analyzed using an Epics XL-MCL flow cytometer (Coulter). Dot plot images were produced with the aid of WinMDI version 2.1.2 by Joseph Trotter (Scripps). Antibodies used were: anti-CD45R-PE (B220), anti-CD8α-RED613, anti-Rat IgG (H + L)-RED613, and anti-CD4-RED613 (GibcoBRL); anti-ly9.1-FITC or anti-ly9.1-biotin, anti-CD8α-FITC, anti-γδ-PE, anti-αβTCR-FITC, and anti-IL-2R-biotin (PharMingen); anti-CD4-PE, anti-αβTCR-PE, and Streptavidin tricolor (Caltag); anti-Ly5.1-FITC (clone #A20). For the analysis of

γδTCR expression in nontransgenic and *NotchIC* transgenic mice, dead cells were excluded on the basis of propidium iodine staining, and 10^6 γδTCR$^+$ cells were analyzed. *TCR*δ$^{-/-}$ mutant mice were analyzed in parallel as a negative control. Data were acquired by FacsVantage flow cytometer (Becton-Dickinson, Mountain View, CA) and analyzed using CellQuest software (Becton-Dickinson).

PCR–RFLP Analysis

PCR–RFLP analysis was performed as previously described (Dudley et al., 1995). Briefly, DNA was isolated from thymocyte samples using standard procedures. V–J regions were amplified in 100 μl PCR reactions containing approximately 10^4 cell equivalents of thymocyte DNA for 35 cycles, using Pfu polymerase (Stratagene) according to the protocol of the manufacturer. PCR products were purified by phenol/chloroform extraction and ethanol precipitation and digested with appropriate restriction enzymes (New England Biolabs) according to the protocol of the manufacturer. Digested products were purified by phenol/chloroform extraction and ethanol precipitation and end-labeled using ^{32}P γATP. Labeled products were purified by phenol/chloroform extraction and ethanol precipitation and run on denaturing polyacrylamide gels (DNA sequencing gels). Gels were dried and radioactivity measured using a PhosphorImager (Molecular Dynamics).

Acknowledgments

Correspondence regarding this paper should be addressed to E. R. We thank Kathryn Anderson, Astar Winoto, and David Raulet for comments on the manuscript; members of the Robey lab for helpful discussions and comments on the manuscript; David Baltimore for *rag1* mutant mice; J. Bluestone and L. Matis for *G8 TCR* transgenic mice; and Peter Schow for expert assistance with flow cytometry. T. W. was supported by a postdoctoral fellowship from the American Cancer Society and a National Institutes of Health training grant. This work was supported by grants to E. R. from the California Cancer Research Coordinating Committee and American Cancer Society.

Received October 18, 1996; revised February 4, 1997.

References

Allison, J., and Lanier, L. (1987). The T-cell antigen receptor gamma gene: rearrangement and cell lineages. Immunol. Today *8*, 293–296.

Allison, J. (1993). Gamma delta T-cell development. Curr. Opin. Immunol. *5*, 241–246.

Beitel, G., Tuck, S., Greenwald, I., and Horvitz, H. (1995). The C. elegans gene lin-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes Dev. *9*, 3149–3162.

Bluestone, J., Cron, R., Barrett, T., Houlden, B., Sperling, A., Dent, A., Hedrick, S., Rellahan, B., and Matis, L. (1991). Repertoire development and ligand specificity of murine γδ T cells. Immunol. Rev. *120*, 5–33.

Bonneville, M., Ishida, I., Itohara, S., Verbeek, S., Berns, A., Kanagawa, O., Haas, W., and Tonegawa, S. (1990). Self-tolerance to transgenic gamma delta T cells by intrathymic inactivation. Nature *344* , 163–165.

Chaffin, K.E., Beals, C.R., Wilkie, T.M., Forbush, K.A., Simon, M.I., and Perlmutter, R.M. (1990). Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosyltransferase. EMBO J. *9*, 3821–3829.

Chien, Y., Iwashima, M., Kaplan, K., Elliot, J., and Davis, M. (1987). A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature *327*, 677–682.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature *375*, 761–766.

Coffman, C., Harris, W., and Kintner, C. (1990). Xotch, the Xenopus homolog of Drosophila notch. Science *249*, 1438–1441.

Coligan, J., Kruisbeek, A., Margulies, D., Shevach, E., and Strober, W. (1994). Current Protocols in Immunology. (New York: John Wiley and Sons, Inc.).

Conlon, R., Reaume, A., and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. Development *121*, 1533–1545.

del Amo, F., Smith, D., Swiatek, P., Gendron-Maguire, M., Greenspan, R., McMahon, A., and Gridley, T. (1992). Expression pattern of Motch, a mouse homolog of Drosophila Notch, suggests an important role in early postimplantation mouse development. Development *115*, 737–744.

Dent, A.L., Matis, L.A., Hooshmand, F., Widacki, S.M., Bluestone, J.A., and Hedrick, S.M. (1990). Self-reactive gamma delta T cells are eliminated in the thymus. Nature *343*, 714–719.

Dudley, E., Girardi, M., Owen, M., and Hayday, A. (1995). αβ and γδ T cells can share a late common precursor. Curr. Biol. *5*, 659–669.

Fehling, H., Krotkova, A., Saint-Ruf, C., and von Boehmer, H. (1995). Crucial role of the pre-T cell receptor α gene in the development of αβ but not γδ T cells. Nature *375*, 795–798.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, N., Hoemann, C., Simard, C., Kozak, C., and Jolicoeur, P. (1996). Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev. *10*, 1930–1944.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3$^-$CD4$^-$CD8$^-$ triple negative adult mouse thymocytes defined by CD44 and CD25 expression. J. Immunol. *150*, 4244–4252.

Hasserjian, R., Aster, J., Davi, D., Weinberg, D., and Sklar, J. (1996). Modulated expression of Notch1 during thymocyte development. Blood *88*, 970–976.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. Cell *64*, 1083–1092.

Kang, J., Baker, J., and Raulet, D. (1995). Evidence that productive rearrangements of TCR γ genes influence the commitment of progenitor cells to differentiate into αβ or γδ T cells. Eur. J. Immunol. *9*, 2706–2709.

Katz, W., Hill, R., Clandinin, T., and Sternberg, P. (1995). Different levels of the C. elegans growth factor LIN-3 promote distinct vulval precursor fates. Cell *82*, 297–307.

Kersh, G., Hooshmand, F., and Hedrick, S. (1995). Efficient maturation of αβ lineage thymocytes to the CD4$^+$CD8$^+$ stage in the absence of TCR-β rearrangement. J. Immunol. *154*, 5706–5714.

Lardelli, M., and Lendahl, U. (1993). Motch A and motch B—two mouse Notch homologues coexpressed in a wide variety of tissues. Exp. Cell Res. *204*, 364–372.

Lardelli, M., Dahlstrand, J., and Lendahl, U. (1994). The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mech. Dev. *46*, 123–136.

Livak, F., Petrie, H.T., Crispe, I.N., and Schatz, D.G. (1995). In-frame TCR δ rearrangements play a critical role in the αβ/γδ lineage decision. Immunity *2*, 617–627.

Lynch, F., and Shevach, E. (1993). γδ T cells promote CD4 and CD8 expression by SCID thymocytes. Int. Immunol. *5*, 991–995.

Mombaerts, P., Clarke, A., Rudnicki, M., Iacomini, J., Itohara, S., Lafaille, J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M., and Tonegawa, S. (1992a). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. Nature *360*, 225–231.

Mombaerts, P., Iacomini, J., Johnson, R., Herrup, K., Tonegawa, S., and Papaioannou, V. (1992b). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maloy, W.L., Coligan, J.E., and Schwartz, R.H. (1987). Differential expression of two distinct T-cell receptors during thymocyte development. Nature *326*, 79–81.

Petrie, H., Scollay, R., and Shortman, K. (1992). Commitment to the

T cell receptor-αβ or -γδ lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. Eur. J. Immunol. *22* , 2185–2188.

Raulet, D., Spencer, D., Hsiang, Y.-H., Goldman, J., Bix, M., Liao, N.-S., Zijlstra, M., Jaenisch, R., and Correa, I. (1991). Control of γδ T cell development. Immunol. Rev. *120*, 185–204.

Robey, E.A., and Fowlkes, B.J. (1994). Selective events in T cell development. Annu. Rev. Immunol. *12*, 675–705.

Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell *87*, 483–492.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H., and von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. Science *266*, 1208–1212.

Schweighoffer, E., and Fowlkes, B. (1996). Positive selection is not required for thymic maturation of transgenic γδ T cells. J. Exp. Med. *183*, 2033–2041.

Shinkai, Y., Rathbun, G., Lam, K., Oltz, E., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A., and Alt, F. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Shores, E., Sharrow, S., Uppenkamp, I., and Singer, A. (1990). T cell receptor-negative thymocytes from SCID mice can be induced to enter the CD4/CD8 differentiation pathway. Eur. J. Immunol. *20*, 69–77.

Sim, G., Olsson, C., and Augustin, A. (1995). Commitment and maintenance of the αβ and γδ T cell lineages. J. Immunol. *154*, 5821–5831.

Spanopoulou, E., Roman, C., Corcoran, L., Schlissel, M., Silver, D., Nemazee, D., Nussenzweig, M., Shinton, S., Hardy, R., and Baltimore, D. (1994). Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. Genes Dev. *8*, 1030–1042.

Stifani, S., Blaumueller, C., Redhead, N., Hill, R., and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nat. Genet. *2*, 119–127.

Swiatek, P., Lindsell, C., del Amo, F., Weinmaster, G., and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. Genes Dev. *8*, 707–719.

Weinmaster, G., Roberts, V.J., and Lemke, G. (1991). A homologue of Drosophila Notch expressed during mammalian development. Development *113*, 199–205.

Weinmaster, G., Roberts, V.J., and Lemke, G. (1992). Notch2: a second mammalian Notch gene. Development *116*, 931–941.

Wilkinson, H., Fitzgerald, K., and Greenwald, I. (1994). Reciprocal changes in expression of the receptor *lin-12* and its ligand *lag-2* prior to commitment in a C. elegans cell fate decision. Cell *79*, 1187–1198.